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Biochemical and Functional Properties of a Palindromic Sequence Motif within the Hepatitis B Virus Enhancer 1

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The hepatitis B virus (HBV) enhancer 1 is a transcriptional element that contributes to the liver-specific regulation of HBV gene expression. We previously identified a novel protein binding site within the enhancer that contains an 8-bp palindromic sequence motif. This motif partially overlaps the binding sites for nuclear factor 1 and hepatocyte nuclear factor 3β (HNF3β). Moreover, we demonstrated that this novel site is recognized by a protein or proteins, tentatively designated as palindrome-binding factor (PBF), that cooperatively interact with HNF3β. In the present work, we have further examined the biochemical and functional attributes of PBF. Protein–DNA interaction studies indicate that three thymidine residues located at the 3′-end of the palindromic sequence motif are important for maximal PBF-binding activity. When protein–DNA complexes were photocrosslinked by exposure to ultraviolet (UV) light, a prominent polypeptide with an apparent molecular mass of 50 kDa was found to associate with the PBF-binding site. Furthermore, transient transfection studies support the hypothesis that PBF contributes to enhancer 1 activity by a combinatorial mechanism that involves at least one other *cis*-acting sequence motif, the HNF3β-binding site.

INTRODUCTION

The human hepatitis B virus (HBV) predominantly infects the hepatocytes of the liver. The clinical manifestations of chronic HBV infection include severe liver disease and cirrhosis (Bader, 1995). To compound these problems, individuals that are chronically infected with HBV are at high risk for developing primary hepatocellular carcinoma (Beasley and Hwang, 1984). To investigate the molecular basis for the pathogenesis of HBV-induced disease, the goal of a considerable amount of work has been to characterize the biochemical mechanisms that contribute to the hepatotropic nature of this virus. Studies have addressed this issue by focusing on liver-specific aspects of HBV gene expression (reviewed in Kosovsky *et al.,* 1998; Yen, 1993). The HBV genome contains four primary translational open reading frames (ORFs) (Fig. 1A), which encode the hepatitis B surface antigen [HBsAg (S ORF)], hepatitis B core and e antigens [HBcAg and HBeAg, respectively (C ORF)], viral polymerase (P ORF), and X protein (X ORF) (Tiollais *et al.,* 1985). The regulation of viral transcription is governed by four promoter elements and two enhancer elements on the HBV genome.

The enhancer 1 element (Fig. 1B) has been shown to play an important role in the overall regulation of HBV gene expression. Enhancer 1 facilitates the activation of the HBV surface antigen (Antonucci and Rutter, 1989; Bulla and Siddiqui, 1988; Hu and Siddiqui, 1991; Su and Yee, 1992), core/pregenomic (Antonucci and Rutter, 1989; Honigwachs *et al.,* 1989; Hu and Siddiqui, 1991; Lopez-Cabrera *et al.,* 1990; Su and Yee, 1992; Zhang and McLachlan, 1994; Zhang *et al.,* 1992), and X (Fukai *et al.,* 1997; Guo *et al.,* 1991; Gustin *et al.,* 1993; Hu and Siddiqui, 1991; Zhang *et al.,* 1992) promoter elements. In addition, studies of HBV gene expression in transgenic mice support the conclusion that enhancer 1 activity *in vivo* contributes to the liver-specific expression of the core/pregenomic promoter (Billet *et al.,* 1995). Work from a number of laboratories further demonstrates that enhancer 1 exhibits liver-specific properties (e.g., see Antonucci and Rutter, 1989; Honigwachs *et al.,* 1989; Jameel and Siddiqui, 1986; Shaul *et al.,* 1985). Enhancer 1 activity is mediated by a complex array of liver-enriched as well as ubiquitous *trans*-acting cellular DNA-binding proteins (Fig. 1B) (Kosovsky *et al.,* 1998; Schaller and Fischer, 1991; Shaul, 1991; Yen, 1993). Furthermore, enhancer 1 appears to be regulated by multiple mechanisms that are dependent on protein–DNA and protein–protein interactions. For example, enhancer 1 activation is facilitated by extracellular signaling through a retinoic acid responsive element (RARE) (Garcia *et al.,* 1993; Huan *et al.,* 1995; Huan and Siddiqui, 1992; Raney *et al.,* 1997), by cooperative interactions (Dikstein *et al.,* 1990a, 1990b; Garcia *et al.,* 1993; Kosovsky *et al.,* 1996; Reinhold *et al.,* 1995), and by *trans*-activation via the HBV X protein (Doria *et al.,* 1995; Faktor and Shaul, 1990; Maguire *et al.,* 1991; Span-

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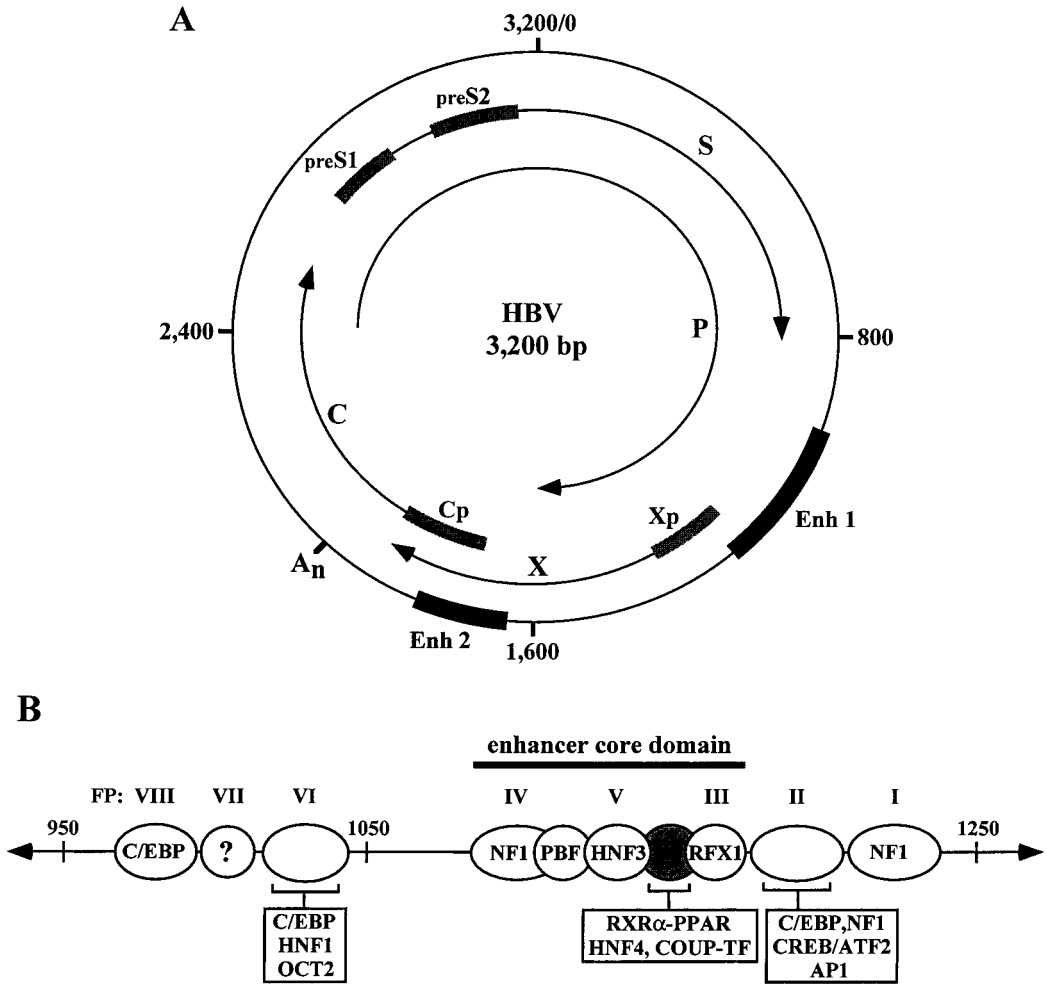

FIG. 1. The hepatitis B virus genome and enhancer 1 element. (A) The viral genome is numbered (0–3200 bp) according to the adw_2 subtype of HBV. S, C, P, and X represent the viral genes encoding the surface antigen, core/e antigen, polymerase, and X proteins, respectively. The HBV promoters (preS1, preS2, Cp, and Xp) and enhancers (Enh 1 and Enh 2) are indicated. A_n, the single polyadenylation site used by all of the HBV RNAs. (B) Protein-binding sites on enhancer 1 are labeled accordingly. Previously defined footprint designations (Patel *et al.,* 1989) are indicated above the corresponding sites. The gray circle represents the HBV RARE.

dau and Lee, 1988). In addition to activation mechanisms, a recent study has shown that enhancer 1 activity is down-regulated by the tumor suppressor protein p53 (Ori *et al.,* 1998).

A number of studies support the conclusion that a central region of enhancer 1 located at nucleotides (nt) 1080–1165 (HBV subtype adw_2), the enhancer core domain (Trujillo *et al.,* 1991), predominantly mediates enhancer 1 activity (Dikstein *et al.,* 1990a; Fukai *et al.,* 1997; Garcia *et al.,* 1993; Guo *et al.,* 1991; Huan and Siddiqui, 1992; Kosovsky *et al.,* 1996; Ori and Shaul, 1995; Trujillo *et al.,* 1991). In addition, several protein binding sites located 3′ of the core domain at nt 1166–1240 appear to provide secondary contributions to enhancer 1 activity (Faktor *et al.,* 1990; Garcia *et al.,* 1993; Guo *et al.,* 1991; Maguire *et al.,* 1991; Trujillo *et al.,* 1991). The enhancer core domain contains two prominent *cis*-acting sequence motifs that serve as binding sites for liver-enriched proteins. Mutational analyses have shown that these liver-specific motifs are crucial for enhancer 1-mediated transcriptional activation (Chen *et al.,* 1994; Fukai *et al.,* 1997; Garcia *et al.,* 1993; Guo *et al.,* 1991; Huan and Siddiqui, 1992; Kosovsky *et al.,* 1996; Ori and Shaul, 1995). These motifs are recognized by one or more isoforms of hepatocyte nuclear factor 3 (HNF3α, β, and/or γ) (Chen *et al.,* 1994; Fukai *et al.,* 1997; Kosovsky *et al.,* 1996; Ori and Shaul, 1995) and liver-enriched members of the hormone nuclear receptor family, which include retinoid X receptor α (RXRα), peroxisome proliferator-activated receptor (PPAR), and HNF4 (Fukai *et al.,* 1997; Garcia *et al.,* 1993; Huan *et al.,* 1995; Huan and Siddiqui, 1992). The HNF3 isoforms are liver-enriched members of the HNF3/*forkhead* protein family (Lai *et al.,* 1993; Weigel and Jackle, 1990), which contains proteins that have been implicated in the regulation of tissue-specific gene expression and developmental processes (e.g., Ang and Rossant, 1994; Hellqvist *et al.,* 1996; Lai *et al.,* 1993; Vallet *et al.,* 1995; Weinstein *et al.,* 1994). Our previous studies demonstrated that HNF3β is the predominant isoform of HNF3 that interacts with the enhancer core domain (Ko-

sovsky *et al.,* 1996). Additionally, we identified a novel protein binding site on enhancer 1 that overlaps the NF1- and HNF3β-binding sites (Kosovsky *et al.,* 1996). This novel site, 5′-TACAAGGCCTTTCTAAG-3′ (nt 1111–1127), spans a region containing an 8-bp perfect palindrome (underlined). Given the palindromic nature of this sequence motif, the cellular factor or factors that bind to this site have been designated as palindrome binding factor (PBF). Moreover, PBF and HNF3β were shown to cooperatively interact with enhancer 1; therefore, HNF3β appears to activate enhancer 1 by cooperatively interacting with at least one other enhancer-binding factor, PBF. The studies described here further characterize the biochemical properties of PBF and its role in the activation of enhancer 1.

RESULTS AND DISCUSSION

To further examine the interaction between PBF and enhancer 1, we analyzed biochemically purified PBF (PBF pool) using the electrophoretic mobility shift assay (EMSA). DNA competition studies were carried out to confirm the specificity of the interaction between PBF and enhancer 1 (Fig. 2A). In the absence of competitor DNA, one distinct complex was formed between a protein or proteins present in the PBF pool and the wild-type PBF oligonucleotide probe (lane 2). When the assay was carried out in the presence of a 100-fold molar excess of the unlabeled wild-type PBF oligonucleotide, the protein–DNA complex was markedly reduced (lane 3). However, an equal amount of nonspecific competitor DNA had no effect on the protein–DNA complex (lane 4). Therefore, the protein–DNA complex results from a specific interaction with the PBF oligonucleotide probe. The specificity of PBF-binding activity is further supported by the DNA affinity purification of PBF, which was carried out in the presence of a high concentration of nonspecific competitor DNA (Kosovsky *et al.,* 1996). We proceeded to carry out mutational analyses of the PBF-binding site to determine the requirements for maximal PBF-binding activity. Point mutations at the 3′-end of the palindromic sequence (TTT to GGG) were found to dramatically reduce PBF-binding activity compared with that observed in the presence of the wild-type PBF probe (Fig. 2B). Additional point mutations within the PBF-binding site, which were analyzed either independently or in conjunction with the TTT to GGG point mutations, did not further reduce PBF-binding activity (data not shown). Therefore, the TTT-to-GGG mutations were used for subsequent biochemical and functional analyses of the PBF-binding site.

UV crosslinking analysis was then used to identify the polypeptide or polypeptides that mediate PBF-binding activity. As shown in Fig. 2C, the PBF pool contains a protein with an apparent molecular mass of 50 kDa that exclusively photocrosslinks to the wild-type PBF-binding site, yet does not bind appreciably to the mutant PBF probe. This finding suggests that a 50-kDa protein present in rat liver nuclei mediates PBF-binding activity. In accordance with these results, SDS–PAGE analysis of the PBF pool revealed the presence of a predominant purified protein that migrates at an apparent molecular mass of 50 kDa (Fig. 2D). Additional proteins that were detected by silver staining exhibited apparent molecular masses of 43, 60, 70, and 86 kDa. Because the UV crosslinking method produces a crosslinked product that is composed of protein and covalently associated oligonucleotide, it is possible that PBF-binding activity is mediated by one of the smaller polypeptides of approximately 43 kDa. This possibility may account for the contribution of the oligonucleotide to the migration of the crosslinked complex. Furthermore, it is possible that PBF-binding activity is mediated by more than one polypeptide. In this case, our results would indicate that only one polypeptide efficiently crosslinked to the labeled probe; therefore, additional studies are necessary to resolve this important issue.

Because protein–DNA interaction studies demonstrated that PBF and HNF3β cooperatively interact with enhancer 1 (Kosovsky *et al.,* 1996), we hypothesized that HNF3β-mediated enhancer activity involves a cooperative interplay between PBF and HNF3β. To address this hypothesis, we carried out functional analyses of the PBF–HNF3β region in the context of the full-length HBV enhancer 1 element (Fig. 3). The TTT-to-GGG mutations described above were incorporated into the PBF sequence motif within the wild-type and HNF3β mutant (V′Luc; Huan and Siddiqui, 1992) enhancer 1 to generate the PBF mutant (P′Luc) and the PBF–HNF3β double mutant (P′V′Luc), respectively (Fig. 3A). The reporter plasmid P′Luc exhibited wild-type levels of enhancer activity, which indicates that mutations within the PBF-binding site alone do not adversely affect enhancer function (Fig. 3B). Because PBF exhibited weak binding to the mutant PBF-binding site (Fig. 2B), we hypothesize that the activity exhibited by P′Luc may be due to wild-type levels of PBF-binding activity in the presence of HNF3β-mediated cooperativity. This hypothesis is consistent with our preliminary studies, which suggest that cooperative-binding occurs in the presence of the mutated PBF-binding site. Therefore, we suggest that enhancer 1 activity is restored to wild-type levels when the PBF mutations are present in conjunction with the wild-type HNF3-binding site. Point mutations within the HNF3β-binding site alone in V′Luc, which abrogate the interaction between HNF3β and enhancer 1 (Kosovsky *et al.,* 1996), reduced enhancer 1 activity to approximately 41% of the wild-type level. When mutations were present within both the PBF- and HNF3β-binding sites in P′V′Luc, enhancer activity was further reduced to approximately 25% of the wild-type level. These results suggest that the PBF-binding site contributes to enhancer 1 activity by a combinatorial mechanism that involves at least one other *cis*-acting sequence motif, the overlapping HNF3β-binding site. Moreover, our prelimi-

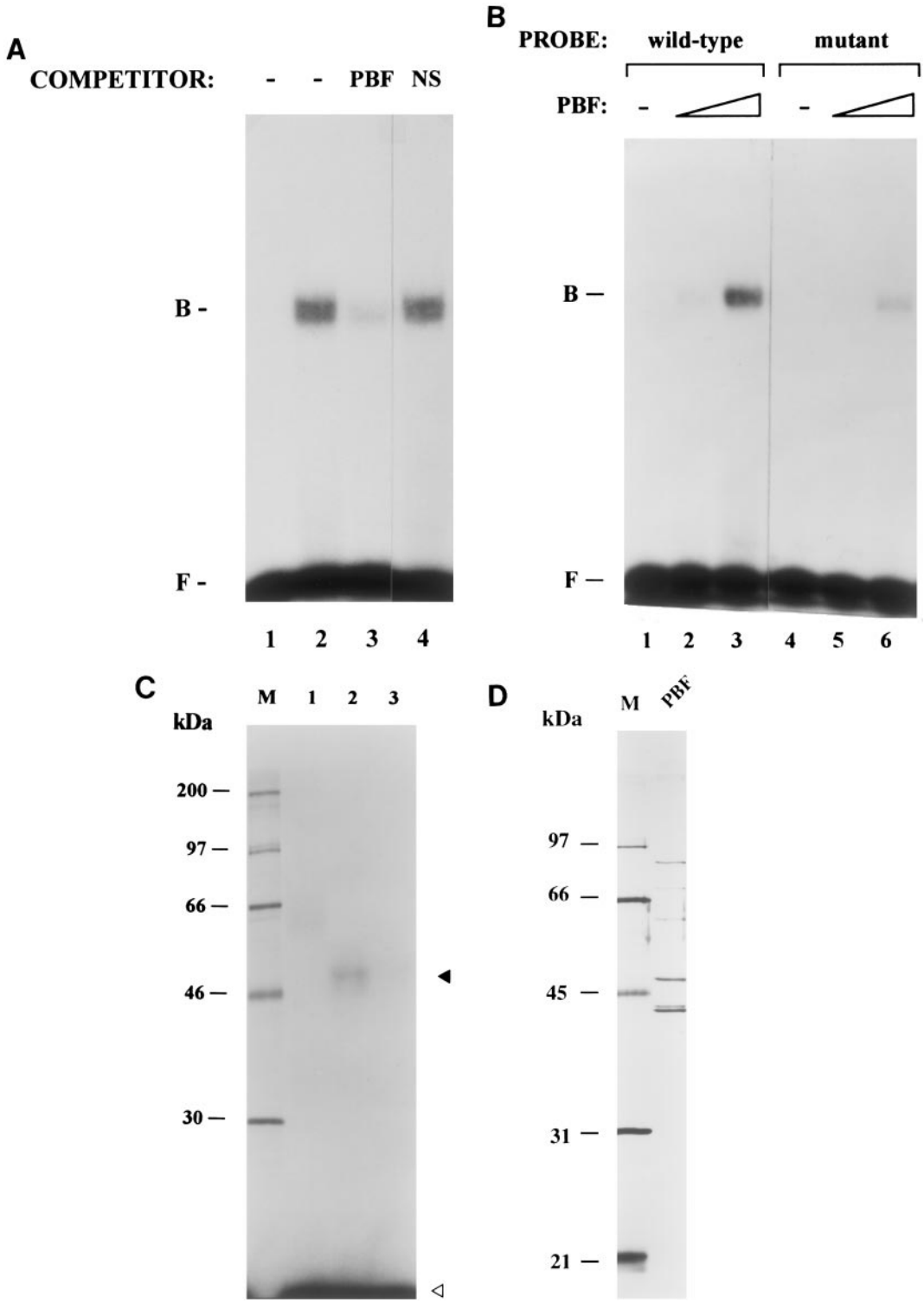

FIG. 2. (A) DNA competition analysis of PBF-binding activity. The EMSA was carried out in the presence of [^{32}P]-labeled PBF oligonucleotide probe. Lane 1, no protein; lanes 2–4, 1 ng of the PBF pool incubated in the presence of the following unlabeled competitor DNAs: lane 2, no competitor; lane 3, 100-fold molar excess of the wild-type PBF oligonucleotide; lane 4, 100-fold molar excess of a nonspecific oligonucleotide (NS). Free probe (F) and bound protein–DNA complexes (B) are indicated. (B) Mutational analysis of PBF-binding activity. Protein samples were incubated in the presence of wild-type (lanes 1–3) or mutant (lanes 4–6) [^{32}P]-labeled PBF oligonucleotide probe and the following: lanes 1 and 4, no addition; lanes 2 and 5, 0.67 ng of the PBF pool; and lanes 3 and 6, 1 ng of the PBF pool. (C) UV crosslinking analysis of the PBF pool. Protein samples were incubated in the presence of wild-type (lanes 1 and 2) or mutant (lane 3) [^{32}P]-labeled PBF oligonucleotide probe. Samples were then analyzed by SDS–PAGE and autoradiography. Lane M, [^{35}S]-labeled molecular mass standards in kilodaltons. Samples contained the respective probe and the following: lane 1, no addition; lanes 2 and 3, 1.8 ng of the PBF pool. The black arrowhead indicates the position of a crosslinked product with an apparent molecular mass of 50 kDa. The white arrowhead indicates the position of free probe. (D) SDS–PAGE analysis of the PBF pool. Proteins were resolved on an SDS–10% polyacrylamide gel and then stained with silver nitrate. The protein sample from the PBF pool (100 μl) was precipitated in the presence of 4 volumes of acetone for 30 min at −70°C. After centrifugation for 10 min at 13,000 rpm, the protein pellet was dried and resuspended in SDS-loading buffer before electrophoresis. Lane M, molecular mass standards. Lane PBF, 65 ng of the PBF pool.

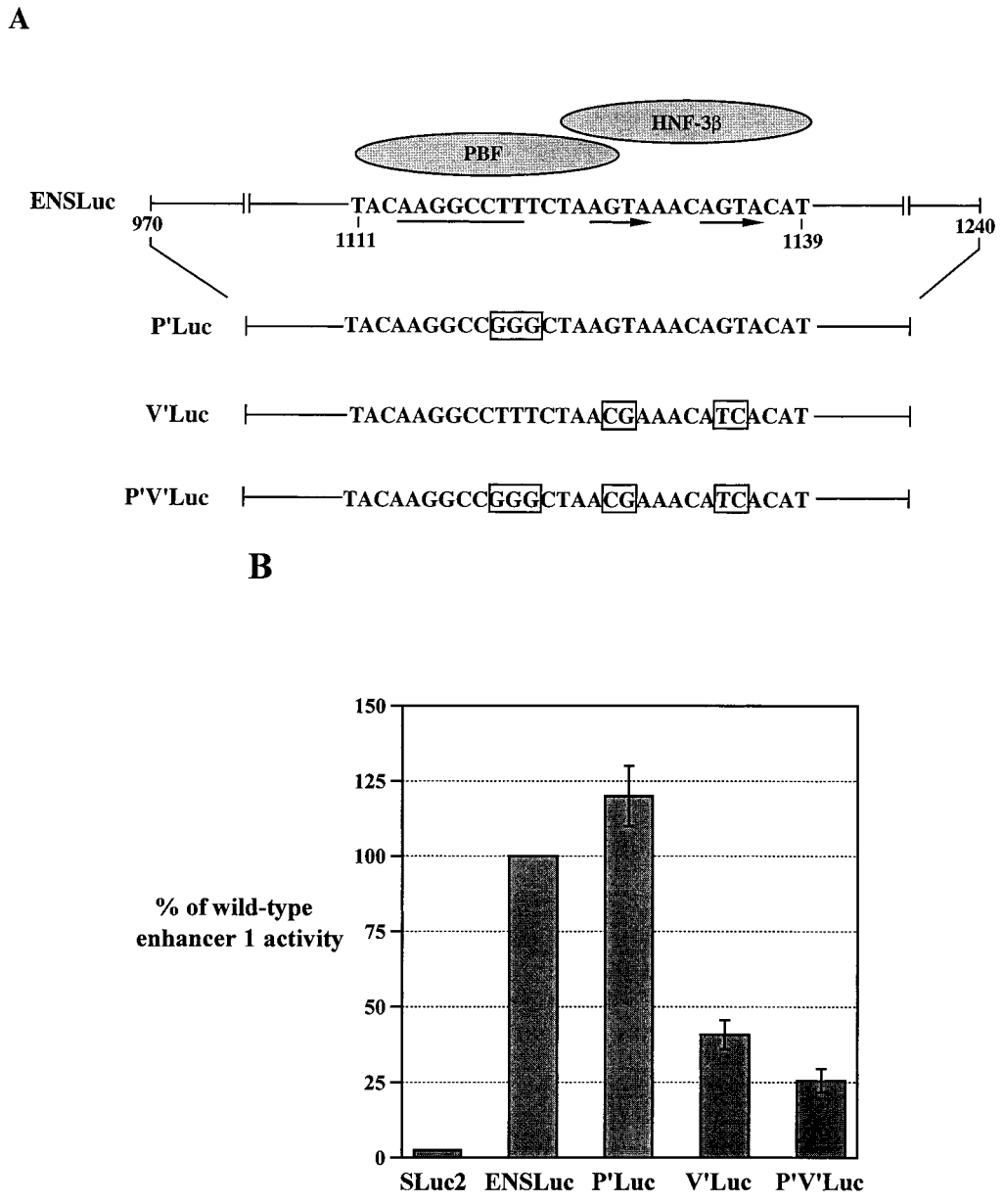

FIG. 3. Functional analysis of enhancer 1 activity. (A) Reporter plasmids contain the following enhancer 1 derivatives upstream of the SV40 early promoter (SV40Ep) and luciferase gene: ENSLuc, wild-type enhancer 1; P′Luc, enhancer 1 containing point mutations in the PBF-binding site; V′Luc, enhancer 1 containing point mutations in the HNF3β-binding site; P′V′Luc, enhancer 1 containing point mutations in the PBF- and HNF3β-binding sites. The thin line and arrows below the PBF–HNF3β region denote the PBF palindromic sequence and AGTA direct repeat, respectively. (B) Transient transfection assays were performed using the human hepatoma cell line Huh-7. Luciferase expression was normalized for transfection efficiency based on the expression of β-galactosidase. Luciferase activity derived from reporter plasmids containing mutated enhancer 1 is expressed as the respective percentage of activity exhibited by the wild-type enhancer 1. The relevant reporter plasmids are indicated below the graph. SLuc2, enhancerless plasmid containing the SV40Ep upstream of the luciferase gene. The mean ± SD values were derived from three independent experiments carried out in triplicate.

nary studies suggest that PBF cooperates with NF1 to further optimize the binding of nuclear proteins to the enhancer.

This study further demonstrates that the HBV enhancer 1 element is regulated by cooperative interactions between multiple *trans*-acting cellular proteins. Although previous work indicated that C/EBP cooperatively interacts with enhancer 1, enhancer activity was found to inversely correlate with the concentration of C/EBP (Dikstein *et al.,* 1990b). Moreover, while the protein binding sites within the HBV RARE–RFX1 region appear to function in a synergistic manner (Garcia *et al.,* 1993), there has been no evidence for cooperative interactions between the *trans*-acting factors that interact within this region. Because HNF3β is a crucial liver-enriched regulator of enhancer 1 (Chen *et al.,* 1994; Fukai *et al.,* 1997; Guo *et al.,* 1991; Huan and Siddiqui, 1992; Kosovsky *et al.,* 1996; Ori and Shaul, 1995; Trujillo *et al.,* 1991), we further

investigated the mechanisms responsible for HNF3β-mediated enhancer activity. In conjunction with our previous findings (Kosovsky *et al.,* 1996), transient transfection studies support the conclusion that HNF3β activates enhancer 1 by cooperatively interacting with PBF. Although HNF3 interacts with multiple sites throughout the HBV genome, PBF-facilitated activity appears to be exclusively associated with enhancer 1. Because HNF3α has been shown to cooperatively interact with NF1 to regulate the albumin enhancer element (Jackson *et al.,* 1993), PBF may function as a bridging factor to facilitate a cooperative interplay between HNF3β and NF1 on the HBV enhancer 1. Furthermore, our previous functional analyses of enhancer 1 deletion mutants demonstrated that the NF1–PBF–HNF3β region (nt 1087–1139) independently exhibits 48% activity compared with the full-length enhancer 1 (Trujillo *et al.,* 1991). Therefore, because the HNF3β-binding site does not exhibit independent activity when present as a monomer (Garcia *et al.,* 1993), the activity exhibited by the NF1–PBF–HNF3 region appears to be mediated by combinatorial effects between the overlapping protein-binding sites within this region.

MATERIALS AND METHODS

Protein purification

The PBF pool was isolated from rat liver nuclei as previously described (Kosovsky *et al.,* 1996). Briefly, rat liver nuclear extract was fractionated using $(NH_4)_2SO_4$ precipitation and heparin–Sepharose chromatography. Protein fractions containing PBF-binding activity were isolated from the heparin–Sepharose pool using two rounds of DNA oligonucleotide affinity chromatography. PBF-binding activity was then pooled and processed as described. The protein concentration of the PBF pool was determined by scanning densitometric analysis of a silver-stained SDS–polyacrylamide gel using the Molecular Dynamics scanning densitometer.

Protein–DNA interaction assays

The EMSA was carried out essentially as described (Kosovsky *et al.,* 1996). Protein–DNA complexes were resolved on 6% native polyacrylamide gels, which were subsequently dried and exposed to x-ray film at −70°C. PBF-binding activity was analyzed using the following DNA oligonucleotides: (1) wild-type PBF, which spans the PBF-binding site on enhancer 1, 5′-CACTTACAAGGCCTTT-CTAAGTAA-3′; (2) mutant PBF, 5′-CACTTACAAGGCCgggCTAAGTAA-3′ (point mutations are shown in lowercase letters); and (3) NS (nonspecific competitor), 5′-GATCCGTCAAAGAATTGGTCTTTG-3′. Oligonucleotide probes were prepared by end-labeling the respective double-stranded DNAs in the presence of T4 polynucleotide kinase and [γ-^{32}P]ATP. Competition analysis was carried out in the presence of 100-fold molar excess of unlabeled competitor DNA.

For UV crosslinking analysis, the PBF pool was incubated in EMSA reaction buffer in the presence of wild-type or mutant PBF [^{32}P]-labeled oligonucleotide probe for 20 min on ice and then exposed to UV light (λ = 254 nm) for 2 min at 4°C. Samples were then supplemented with SDS-loading buffer and resolved on an SDS–10% polyacrylamide gel, which was subsequently dried and exposed to x-ray film at −70°C. SDS–PAGE and silver staining were carried out as described (Kosovsky *et al.,* 1996).

Transient transfection assay

To generate reporter plasmids P′Luc and P′V′Luc for transient transfection analyses (Fig. 3A), point mutations were incorporated into the PBF–HNF3β region within enhancer 1 using the QuikChange site-directed mutagenesis kit according to the manufacturer (Stratagene). DNA sequencing analysis was performed to confirm the presence of the mutations. Reporter plasmids contained wild-type or mutant enhancer 1 cloned upstream of the simian virus 40 early promoter and luciferase gene. Transient transfection assays were carried out in the human hepatoma cell line Huh-7 essentially as described (Kosovsky *et al.,* 1996). Cell monolayers were transfected with 2.0 μg of luciferase reporter plasmid and 2 μg of a β-galactosidase expression plasmid using the calcium phosphate precipitation method. The cells were incubated for 48 h in the presence of culture medium containing 10% FCS, harvested, and then analyzed for luciferase and β-galactosidase activity.

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